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The contribution of CYP2C gene subfamily involved in epoxygenase pathway of arachidonic acids metabolism to hypertension susceptibility in Russian population

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ABSTRACT

Numerous studies demonstrated an importance of cytochrome P-450 epoxygenase pathway of arachidonic acids metabolism for the pathogenesis of essential hypertension (EH). The present study was designed to investigate whether common single-nucleotide polymorphisms (SNP) of CYP2C gene subfamily such as *CYP2C8* (rs7909236 and rs1934953), *CYP2C9* (rs9332242), and *CYP2C19* (rs4244285) are associated with susceptibility to EH in Russian population. A total of 816 unrelated Russian individuals comprising 425 EH patients and 391 normotensive controls were included into the study. Genotyping of SNPs was performed using the MassARRAY 4 system. SNP rs7909236 of *CYP2C8* was significantly associated with increased risk of EH (OR adjusted for sex and age was 2.99 95% CI 1.39–6.44, $P = 0.005$). SNPs rs1934953 *CYP2C8* and rs4244285 of *CYP2C19* showed association with EH risk but at a borderline statistical significance ($P \leq 0.04$). Combination of genotypes *CYP2C8* rs7909236 TT and *CYP2C19* rs4244285 GG was associated with increased EH risk (OR 3.34 95%CI 1.48–7.51, $P = 0.004$). Genotype–phenotype correlation analysis showed that the levels of *CYP2C8* mRNA were significantly correlated with SNP rs7909236 ($P = 0.01$). *in silico* functional prediction analysis revealed the functionality of majority of investigated SNPs. Thus, genes of CYP2C subfamily are important genetic determinants of susceptibility to essential hypertension in Russians.

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Introduction

Hypertension is a complex multifactorial disease with high worldwide prevalence and associated increased rates of morbidity, mortality, and disability from cardiovascular and cerebrovascular diseases (1, 2). Despite considerable research, the etiology of hypertension in most cases remains elusive, that is, the reason to define the disease as essential hypertension (EH). Although, genome-wide association scans and candidate gene studies have successfully identified a number of common genetic variants influencing blood pressure and hypertension susceptibility (3–7), nevertheless, identification of new potential genes and pathways related to disease pathogenesis remains a matter of great interest.

According to the present available evidence, alterations in the metabolism of arachidonic acid (AA) and other polyunsaturated fatty acids are involved into the pathogenesis of hypertension and other cardiovascular diseases (8–10). Incorporated into membrane phospholipids, AA is hydrolyzed by a Ca^{2+} -activated phospholipase A_2 upon endogenous stimuli and oxidized by cytochrome P450 epoxygenases to form epoxyeicosatrienoic acids (EETs), potent lipid mediators with a wide range of biological events in the cardiovascular system (11, 12). Functional effects

of EETs are realized through activating receptor-mediated signaling pathways and ion channels (12). A number of preclinical and genetic epidemiologic studies demonstrated that CYP-derived epoxyeicosatrienoic acids possess potent vasodilatory, angiogenic, and anti-inflammatory properties in the heart, vasculature, and kidney and that genetically determined alterations in the metabolism of EETs may play an important role in the pathophysiology of many cardiovascular disorders, including hypertension and its complications (13, 14).

Cytochrome P450 2J (*CYP2J2*) and 2C (*CYP2C8*, *CYP2C9* and *CYP2C19*) are the two major subfamilies of CYP enzymes responsible for the biosynthesis of EETs in the cardiovascular system (15, 16). Interestingly, experimental studies demonstrated that increased expression of endothelial CYP2 epoxygenases lowers blood pressure and attenuates hypertension-induced renal injury in mice (17). Taken into account that genetically determined differences in the expression or activity of these enzymes could explain interindividual risks in developing hypertension, exploitation of single-nucleotide polymorphisms (SNP) in genes encoding these enzymes is become an attractive option for examining genetic etiology of essential hypertension. Pursuing this interest,

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several genetic association studies have been undertaken to investigate the relationship between SNPs in CYP epoxygenase genes and hypertension susceptibility in different human populations. However, no association studies of CYP2C and CYP2J gene subfamilies have been so far undertaken on the animal model of human hypertension. The relationship of polymorphisms of CYP2C and CYP2J gene subfamilies and essential hypertension risk has been investigated in various races and ethnicities including African-Americans (18, 19), Chinese (20, 21, 22), Korean (23), and Arab (24, 25) populations. A few genetic association studies investigated CYP2C and CYP2J2 gene subfamilies SNPs and hypertension risk have been done in European populations (18, 26, 27). However, these studies have yielded controversial results and show that the pathogenetic role of CYP2C and CYP2J2 gene subfamilies should be clarified by independent studies in different populations. Our previous study in Russians has found a strong association between promoter polymorphism -76G>T (rs890293) of the *CYP2J2* gene and the risk of essential hypertension (26) highlighting the importance of P-450 epoxygenase pathway of arachidonic acid metabolism in the pathogenesis of hypertension in our population. Therefore, the present study was designed to investigate whether common SNPs of CYP2C subfamily genes such as *CYP2C8* (rs7909236 and rs1934953), *CYP2C9* (rs9332242), and *CYP2C19* (rs4244285) are associated with susceptibility to essential hypertension in Russian population.

Methods

Study Population

A total of 816 unrelated Russian individuals comprising 425 patients with essential hypertension EH and 391 normotensive controls were selected from the study samples recruited in our previous studies (26, 28, 29). The study protocol was approved by Ethical Review Committee of Kursk State Medical University, and written informed consent was obtained from each participant before the study. Diagnosis of essential hypertension was established by qualified cardiologists according to WHO criteria. All hypertensive patients had no clinical signs, symptoms, and laboratory findings suggestive of secondary hypertension. As can be seen from Table 1, patients with EH were matched to healthy controls on sex and age ($P > 0.05$). A percentage of positive family history of hypertension was significantly greater in hypertensive patients versus normotensive controls. No differences were found between the groups regarding to other baseline characteristics.

Genetic analysis

Genomic DNA was isolated from venous blood samples using a standard phenol/chloroform procedure. Genes encoding

CYP2C subfamily epoxygenases of responsible for the metabolism of arachidonic acid into vasoactive eicosanoids were selected based on their involvement in the pathway using KEGG PATHWAY (www.genome.jp/kegg/pathway.html), Reactome Pathway (www.reactome.org), and PharmGKB (www.pharmgkb.org) databases. Four common SNPs of the CYP2C gene subfamily as *CYP2C8* (rs7909236, rs1934953), *CYP2C9* (rs9332242), and *CYP2C19* (rs4244285) were selected for this study. Genotyping of SNPs was performed using the MassARRAY 4 system (Agena Bioscience Inc, San Diego, CA, USA) at the Core Facility “Medical Genomics” in the Research Institute of Medical Genetics (Tomsk, Russia). We failed to genotype eight controls and four cases EH patients because of low quality of their DNA samples.

To evaluate genotype–phenotype correlations, we used the genotype and mRNA expression data available for 270 HapMap subjects from four populations and SNPexp v1.2 online tool (<http://app3.titan.uio.no/biotools/tool.php?app=snpexp>). The functionality of selected SNPs was also assessed *in silico* by the SNP function prediction tool developed by Xu and Taylor (30) and available online at the SNPinfo Web Server (<https://snpinf.niehs.nih.gov/snpinf/snpfunc.htm>).

Data Analysis

Allele frequencies were estimated by the gene counting method, and the chi-square test was used to identify significant departures from Hardy–Weinberg equilibrium. The distributions of the genotypes and their combinations between patients and controls were evaluated by logistic regression analysis. Categorical variables such gender and family history of hypertension were also compared by using the chi-square test. These statistics were calculated by using STATISTICA software for Windows 10.0 (StatSoft Inc., Tulsa, OK, USA) and the SNPassoc package for R. The association between genotypes and EH risk was measured by multiple logistic regression analysis to calculate odds ratios (OR) with 95% confidence intervals (CI) and adjusted for age and gender. Pairwise gene–gene interactions were evaluated by log-likelihood ratio test (LRT) assuming codominant, dominant, and recessive models and adjusted for age and gender (31). Generalized linear model (GLM) was used for the genotype–phenotype correlation analysis to evaluate the differences in the relative mRNA expression levels among carriers with different genotypes using data from 270 HapMap subjects.

Results

No departure from Hardy–Weinberg equilibrium for investigated SNPs was noted in both EH and control groups ($P > 0.05$). Allele and genotype frequencies in the studied groups were compatible with those reported in European populations. Table 2 shows genotype and allele frequencies of CYP2C subfamily gene polymorphisms in both case and control groups. Variant allele and genotypes of *CYP2C8* rs7909236 were found to be significantly associated with increased risk of essential hypertension ($P \leq 0.001$). As can be seen from Table 2, association between genotypes *CYP2C8* and EH risk remained statistically significant after adjustment for age and sex ($P = 0.005$). Moreover, allele C (OR =

Table 1. Baseline characteristics of the study groups.

Baseline characteristics	Controls, <i>n</i> = 391	EH patients, <i>n</i> = 425	<i>P</i> -value
Age, mean \pm standard deviation	58.9 \pm 8.4	59.0 \pm 8.3	0.84
Males, <i>n</i> (%)	228 (58.3)	261 (61.4)	0.37
Body mass index (kg/m ²), mean \pm standard deviation	27.5 \pm 7.7	28.2 \pm 9.4	0.24
Positive family history of hypertension, <i>n</i> (%)	183 (46.8)	251 (59.1)	0.001

Bolded is statistically significant *P* value.

Table 2. Genotype and allele frequencies of CYP2C subfamily gene polymorphisms in hypertensive patients and controls.

Gene, polymorphism	Genotype, allele	Controls, n (%) ¹	EH patients, n (%) ¹	P-value OR (95% CI) ²	P-value adjOR (95% CI) ³
CYP2C8, G > T (rs7909236)	GG	262 (67.0)	242 (56.9)	0.001	0.005
	GT	118 (30.2)	151 (35.5)	1.27 (0.95–1.71)	1.38 (0.99–1.93)
	TT	11 (2.8)	32 (7.5)	2.81 (1.40–5.66)	2.99 (1.39–6.44)
	T	0.179	0.253	0.0003	-
CYP2C8, T > C (rs1934953)	TT	181 (46.3)	178 (41.9)	1.55 (1.22–1.97)	0.083
	TC	181 (46.3)	197 (46.4)	0.084	1.09 (0.82–1.46)
	CC	29 (7.4)	50 (11.8)	1.11 (0.83–1.48)	1.76 (1.06–2.90)
	C	0.306	0.349	1.75 (1.06–2.90)	-
	CC	305 (79.6)	337 (80.0)	0.061.22 (0.99–1.50)	0.568
CYP2C9, C > G (rs9332242)	CG	71 (18.5)	80 (19.0)	0.558	1.03 (0.72–1.47)
	GG	7 (1.8)	4 (1.0)	1.02 (0.71–1.45)	1.03 (0.72–1.47)
	G	0.111	0.105	0.52 (0.15–1.78)	0.52 (0.15–1.81)
	G	0.111	0.105	0.677	-
CYP2C19, G > A (rs4244285)	GG	305 (78.0)	341 (80.2)	0.94 (0.68–1.28)	0.117
	GA	74 (18.9)	80 (18.8)	0.119	0.95 (0.65–1.39)
	AA	11 (3.1)	4 (0.9)	0.99 (0.70–1.41)	0.29 (0.08–1.05)
	A	0.123	0.104	0.35 (0.12–1.06)	-
	A	0.123	0.104	0.21	-
				0.82 (0.61–1.12)	

¹ Absolute number and percentage of individuals with particular genotype.

² Odds ratio with 95% confidence intervals (codominant genetic model).

³ Odds ratio with 95% confidence intervals adjusted for age and gender with two degrees of freedom.

Bolded is statistically significant *P* value.

1.22 95%CI 0.99–1.50, *P* = 0.06) and genotype CC (OR = 1.66 95% CI 1.03–2.69, *P* = 0.035, recessive genetic model) of CYP2C8 rs1934953 were also associated with increased hypertension risk, but at a borderline statistical significance. However, odds ratio of EH risk in the carriers of CYP2C8 CC remained significant after adjustment for age and sex (OR = 1.68 95%CI 1.04–2.72, *P* = 0.031). In addition, SNP rs4244285 of CYP2C19 tended to be associated with a decreased risk of EH: Adjusted odds ratio for sex and age in the carriers of genotype AA was 0.29 (95%CI 0.08–1.05, *P* = 0.04).

Then, we applied a log-likelihood ratio in order to assess joint effects of CYP2C subfamily gene polymorphisms on EH risk. Table 3 shows data on the analysis for epistatic interactions between CYP2C subfamily gene polymorphisms in essential hypertension, as evaluated by SNPAssoc package for R. Although, no statistically significant epistatic interactions between the genes were observed, the crude effect of rs1934953 on the EH risk was noted (*P* = 0.033, recessive genetic model). Further

analysis for gene–gene interactions (Table 4) observed that five genotype combinations were associated with increased risk of hypertension. However, the only genotype combination CYP2C8 rs7909236 TT × CYP2C19 rs4244285 GG was significantly associated with EH risk after adjustment for multiple testing (*P* = 0.004).

Genotype–phenotype correlation analysis for CYP2C subfamily gene polymorphisms was performed using the data on both mRNA expression levels and genotypes for these genes obtained from 270 HapMap individuals. The relative expression levels of CYP2C subfamily genes mRNA by different genotypes are shown in Figure 1(A, B, C and D). The levels of CYP2C8 mRNA were significantly correlated with SNP rs2228099 (*P* = 0.01, Figure 1A) at dominant genetic model, as evaluated by GLM. No significant genotype–phenotype correlations were found for other investigated SNPs. *in silico* functional analysis performed by the SNP function prediction tool observed a regulatory potential for SNP rs1934953 of the

Table 3. Epistatic interactions between CYP2C subfamily gene polymorphisms in essential hypertension. (Gene–gene interactions are evaluated by SNPAssoc package for R [González JR et al, 2007]).

SNPs	Genetic models	CYP2C8 (rs7909236)	CYP2C8 (rs1934953)	CYP2C9 (rs9332242)	CYP2C19 (rs4244285)
CYP2C8 (rs7909236)	Codominant	-	-	-	-
	Dominant	-	-	-	-
	Recessive	-	-	-	-
	Overdominant	-	-	-	-
CYP2C8 (rs1934953)	Codominant	-	0.086	0.052	-
	Dominant	-	0.236	0.663	-
	Recessive	-	0.033	-	-
	Overdominant	-	0.927	0.381	-
CYP2C9 (rs9332242)	Codominant	-	0.745	0.572	-
	Dominant	-	0.970	0.920	-
	Recessive	-	0.488	0.295	-
	Overdominant	-	0.917	0.835	-
CYP2C19 (rs4244285)	Codominant	-	-	-	-
	Dominant	-	-	-	-
	Recessive	-	-	-	-
	Overdominant	-	-	-	-

The upper part of the matrix contains the *P* values for epistatic interactions evaluated by log-likelihood ratio (LRT) test. The diagonal contains the *P* values from LRT for the crude effect of each SNP. The lower triangle contains the *P* values from LRT comparing the two-SNP additive likelihood to the best of the single-SNP models. Bolded is statistically significant *P* value for SNP–SNP interactions. *P* values are adjusted for age and gender.

Table 4. Overall associations between genotype combinations of CYP2C subfamily gene SNPs and essential hypertension risk.

Genotype combination	Controls, <i>n</i> (%)	EH patients, <i>n</i> (%)	<i>P</i> -value OR (95% CI) ¹
1 <i>CYP2C8</i> rs7909236 TT × <i>CYP2C8</i> rs1934953 CC	10 (2.9)	22 (7.2)	0.01 2.57 (1.20–5.52)
2 <i>CYP2C8</i> rs7909236 GT × <i>CYP2C9</i> rs9332242 CG	13 (3.9)	23 (7.6)	0.04 2.04 (1.01–4.10)
3 <i>CYP2C8</i> rs7909236 TT × <i>CYP2C9</i> rs9332242 GG	10 (3.0)	20 (6.6)	0.03 2.30 (1.06–5.00)
4 <i>CYP2C8</i> rs7909236 TT × <i>CYP2C19</i> rs4244285 GG	8 (2.5)	21 (8.0)	0.004 3.34 (1.48–7.51)
5 <i>CYP2C8</i> rs1934953 CC × <i>CYP2C19</i> rs4244285 GG	27 (7.5)	42 (12.8)	0.02 1.81 (1.09–3.01)

¹ Crude odds ratio with 95% confidence intervals;

Bolded is statistically significant genotype combination after adjustment for multiple testing.

CYP2C8 gene (Figure 1E it is indicated by right red box). A SNP rs9332242 is located at binding sites for mRNA. Furthermore, SNP rs7909236 is located at binding site for a number of transcription factors suggesting a functional significance of this polymorphism of *CYP2C8*.

Discussion

In the present study, we investigated whether common polymorphisms of *CYP2C8*, *CYP2C9*, and *CYP2C19*, genes involved into the epoxygenase pathway arachidonic acids metabolism, are associated with susceptibility to essential hypertension in Russian population. We found that SNPs rs7909236 and rs1934953 of the *CYP2C8* gene were significantly associated with increased risk of essential hypertension, whereas SNP rs4244285 of *CYP2C19* tended to be associated with decreased risk of the disease. Analysis for gene–gene interactions revealed that combination of genotypes *CYP2C8* rs7909236 TT and *CYP2C19* rs4244285 GG was significantly associated with increased risk of essential hypertension. Genotype–phenotype correlation analysis showed that the levels of *CYP2C8* mRNA were significantly correlated with SNP rs7909236. Functional prediction analysis performed *in silico* revealed the functionality of majority of investigated SNPs: a regulatory potential observed for SNP rs1934953 of *CYP2C8*, SNP rs9332242 of *CYP2C8* is located at binding sites for mRNA, and SNP rs7909236 of *CYP2C8* is located at binding site for multiple of transcription factors.

To our knowledge, the present study was the first to show that the investigated polymorphisms such as rs7909236 and rs1934953 of *CYP2C8* and also rs4244285 of *CYP2C19* could be novel genetic markers of susceptibility to essential hypertension. We also found for the first time that synergistic interactions between polymorphic genes of *CYP2C* subfamily, at least between *CYP2C8* rs7909236 and *CYP2C19* rs4244285 loci, contribute to the increased disease risk. However, the key finding was an increased risk for EH in carriers of the variant T alleles of rs7909236. *CYP2C8* is a cytochrome P450 enzyme possessing epoxygenase activity and catalyzing an NADPH- and oxygen-dependent conversion of arachidonic acid to a cis-epoxyeicosatrienoic acid. The enzyme plays a major role in the metabolism of therapeutically important drugs exhibiting large interindividual differences in their pharmacokinetics (32). In fact, little is known about how the expression of the *CYP2C8* gene is regulated. It is known that rs7909236 (-271C>A, *CYP2C8*1B*) is a promoter SNP located at -271

from the translational start of the *CYP2C8* gene, and the variant allele has been suggested to result in the creation of a C/EBPalpha transcription factor consensus sequence and associated with increased transcription factor binding and promoter activity (32, 33). However, this is contrary to our finding obtained on the HapMap data. We found that the levels of *CYP2C8* mRNA were decreased in the carriers of genotype GT suggesting a downregulation effect of this variant on the gene expression. Indeed, polymorphism rs7909236 of *CYP2C8* could be the target for a number of transcription factors, as we identified *in silico* by the function prediction tool. This means that the expression of *CYP2C8* is controlled by diverse regulatory mechanisms such as inducible activation by transcription factors, each of which bind to specific regulatory elements in this 5'-upstream region of the gene. We did not find effects of other SNPs investigated in this study on the expression levels of corresponding genes. Moreover, no any functional studies were found in the literature regarding to functional significance for that SNPs. This makes further interpretation of association of these SNPs with hypertension phenotype difficult.

Given that *CYP2C*-derived EETs are potent vasodilators, persons with diminished capacity to synthesize EETs due to the downregulation of *CYP2C8* expression (i.e., carriers of the -271T allele of rs7909236) might be more susceptible to essential hypertension. Based on the reviewed literature (9, 10, 11, 14), we proposed the mechanisms by which plausible deficiency of *CYP2C*-derived EETs in carriers of the -271T allele of *CYP2C8* might cause hypertension include: a) decreased vasorelaxation and increased peripheral vascular resistance due to diminished ion channel-dependent activation and insufficiency to stimulate the formation and release of natural vasodilators (action as an endothelial derived hyperpolarizing factor), b) increased cardiac contractility and heart rate, c) increased cortical renin release and decreased sodium excretion in kidneys due activating epithelial sodium channel, and d) promoted both apoptotic and oxidative vascular injury. Certainly, it is crucial that, before drawing definitive conclusions about the mechanisms proposed above, experimental and clinical studies utilizing cutting edge molecular and biochemical technologies are required to substantiate genotype–phenotype relationships in the epoxygenase pathway of AA metabolism underlying hypertension pathogenesis.

The present study has some limitations. A majority of the associations of SNPs with hypertension risk were not strong,

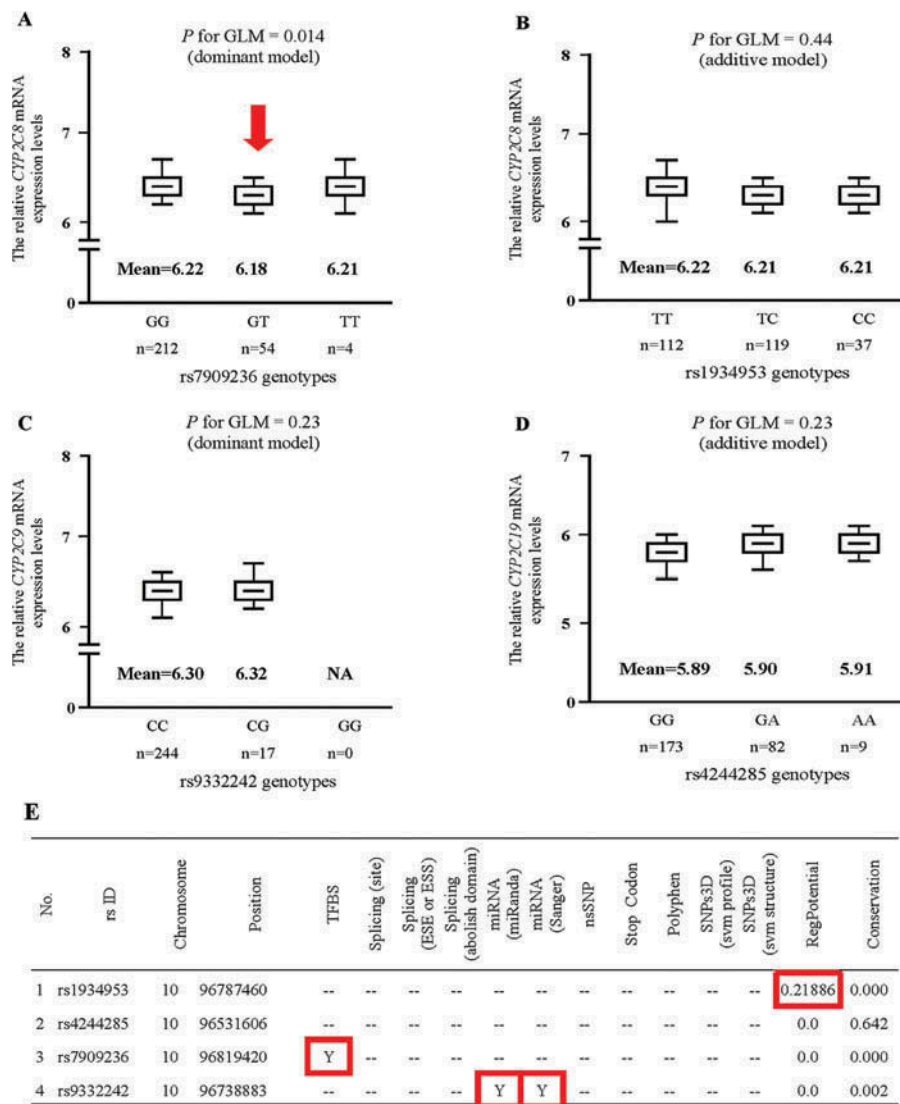


Figure 1. Functional analysis of CYP2C subfamily gene polymorphisms. A, B, C, and D. The relative expression levels of CYP2C subfamily genes mRNA by different genotypes in 270 HapMap subjects from four populations analyzed by the HumanWG-6 Expression BeadChip and evaluated by SNPexp v1.2 online tool (Norwegian PSC Research Center, <http://app3.titan.uio.no/biotools/tool.php?app=snpexp>). Red arrow shows significant effects of SNP on gene expression levels. The effects of SNPs on mRNA levels of corresponding genes are evaluated by generalized linear models (GLM). E. SNP function prediction results evaluated by FuncPred online tool (National Institute of Environmental Health Sciences, <https://snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm>). NA, not available; TFBS, Transcription Factor Binding Sites; ESE, Exonic Splicing Enhancer; ESS, Exonic Splicing Silencer; Polyphen, predicted damaging nsSNPs; RegPotential, Regulatory Potential Score; Y, Yes; N, No.

thereby showing small-to-modest effects of these genes on disease phenotype. Because a limited number of SNPs were selected for this study, our findings do not allow any definitive conclusion yet to be made on the comprehensive contribution of the genes to hypertension susceptibility. This means that the results of our study require further investigation in other population-based studies to address issues discussed above, but these studies will need to be substantially larger than the present investigation. It is important to note that since essential hypertension is a multifactorial disease determined by a complex interplay between genetic and environmental factors, further genetic studies should take into account disease-associated environmental risk factors to better understand the molecular mechanism of the disease development.

Nevertheless, a preliminary conclusion has to be made that polymorphic genes of CYP2C subfamily are important determinants of genetic susceptibility to essential hypertension. An

association between polymorphisms of these genes and hypertension demonstrates the importance of the epoxygenase pathway in the metabolism of arachidonic acids for the disease pathogenesis. Understanding the relationships between altered *CYP2C9* gene expression and hypertension pathophysiology should suggest specific targeted interventions that will expand the therapeutic options for hypertensive individuals in future. For instance, the modulation of EET pathway through stimulation of residual enzyme activity represents a promising pharmacological target that may improve the clinical management of hypertensive patients and healthy individuals at high cardiovascular risk.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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